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Studies concerning the correlation of nodulation and nitrogen-fixation by various strains of Rhizobium leguminosarum

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STUDIES CONCERNING THE CORRELATION OF NODULATION AND NITROGEN-FIXATION BY VARIOUS STRAINS OF RHIZOBIUM LEGUMINOSARUM

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INTRODUCTION

Nitrogen is one of the most essential elements of plant growth. Each year over four million tons of this element are removed from the arable soils of the United States. Some of the loss is due to natural leaching processes but the greater part goes into the cellular construction of plants. The problem of replacing this enormous loss of soil nitrogen is now recognized in all systems of agriculture.

Until recently, natural deposits of ammonium sulphate and sodium nitrate have been utilized as the principal sources of nitrogen. These salts are costly to the agriculturist and the supply is in great danger of exhaustion. Within the last few years German workers have perfected a technique for the chemical fixation of atmospheric nitrogen and the products have quickly found a place in the manufacture of nitrogenous fertilizers. There is little danger of exhausting the supply of atmospheric nitrogen, necessary for the process, because natural replacement from the upper strata of the earth's atmosphere is practically unlimited. Due to lower production and transportation costs and better results, the commercial fixation of nitrogen and the manufacture of "synthetic" fertilizer now threatens to displace the natural product.

Although the application of commercial methods to chemical fixation is a recent development, natural nitrogen fixation in the field has long been recognized as an important factor in soil fertility. Early workers in agriculture recommend the use of the Leguminosae to invigorate worn-out, unproductive soils. In the late 19th century scientists

discovered that leguminous plants could store large amounts of nitrogen, especially when grown in soils containing minute amounts of this element. Between 1866 and 1900 Wornin (1866), Wigand (1887), Hellriegel and Wilfarth (1888), Schloessing and Laurent (1892) and Beijerinck (1888) conclusively demonstrated that certain bacteria infect and become established in the roots of the legumes. These organisms, now classified as Rhizobium leguminosarum, when present in the nodules of legume plants, possess the power of fixing atmospheric nitrogen. The process is referred to as Symbiotic Nitrogen Fixation.

The symbiotic fixation of nitrogen in the Leguminosae has made these crops economically important in agricultural practice. Nitrogen fixing bacteria may be classed as potential fertilizer, if properly cultivated with the legumes. A German laboratory first realized the economic value of symbiotic nitrogen-fixing bacteria in the artificial inoculation of legumes and prepared pure cultures of the organisms for commercial distribution. The product appeared in the United States in 1896. American laboratories soon entered the competition and by 1910 there were approximately 10 commercial laboratories preparing and distributing cultures. The quality of the early cultures was variable, some being good ("containing viable bacteria capable of producing infection and accumulating available nitrogen in a specific plant or group of plants"), others absolutely worthless, and the balance somewhere between these two limits.

For the past 15 years, methods employed by various investigators in the study of culture purity and nitrogen fixing ability have remained unchanged. Final judgment of nitrogen fixing ability of the cultures is made upon the theoretical supposition that there is a definite relationship between the number of nodules and the amount of nitrogen fixed in the plant. A search of the literature fails to reveal any experimental data which accurately substantiates the above assumption.

Recent studies at the Wisconsin Experiment Station clearly demonstrate significant physiological differences in the nitrogen requirements of various strains of the legume bacteria. Some strains produce nodules, fix nitrogen and give to the plant an additional quantity of this element in available form; other strains produce nodules, fix nitrogen and immediately utilize the product for cell metabolism. The plant receives no benefit from the latter cultures. The writer assumes the true index of culture value to be nitrogen increase in the plant, and, although heavy nodulation may correlate with this index, there are many instances in which the correllation is entirely lacking. Farmers are not interested in the number of nodules on the roots of the legumes, but in the size and quality of the crop yield (largely dependent upon available nitrogen) and the amount of nitrogen to be added to their land by turning under the crop residue.

The writer is of the opinion that too much attention has been given to nodulation efficiency of legume bacteria and too little to their actual ability to fix atmospheric nitrogen and make it available for plant

use. The experimental work reported in this paper represents a study of methods and technique for a new laboratory procedure for the quantitive study of strain variation, and suggests a practical application of the the method in the testing of commercial legume cultures. Judgment of the quality of the cultures is made by actual measurement of nitrogen accumulation in the infected plant growing under controlled conditions. Nodulating, non-fixing strains can be detected and subsequently eliminated as sources of commercial inoculum, and their relative nitrogen-fixing powers can be studied. The suggested method allows accurate scientific selection of highly efficient culture for use in the field.

The writer believes that available methods and technique are inadequate for regulatory testing and must be supplemented by more exact measurements of fixed nitrogen. The experimental data herein reported provide valuable additional information in regard to the bacteriological examination of commercial legume cultures.

Historical Review

Information on various phases of symbiotic nitrogen fixation has accumulated rapidly since Hellriegel and Wilfarth (1888) discovered that bacteria present in the root nodules of leguminous plants were able to fix atmospheric nitrogen. Beijerinck (1888) successfully isolated the organism in pure culture and studied its morphological and physiological characteristics. He was the first investigator to give

these bacteria a scientific name, which has changed several times in the evolution of scientific nomenclature. The present system of classification gives them the name, Rhizobium leguminosarum. An extensive review of the literature on symbiotic nitrogen fixation is omitted in this discussion because adequate reviews are furnished by Jacobitz (1901), Hiltner (1904), Vögel (1906), Voorhees and Lipman (1907), Löhnis (1910) and Glöbel (1926). Burrill and Hansen (1917) present a long bibliography of the entire problem prior to 1917. Whiting (1917) extensively discusses the biochemistry of the subject. Researches during the last decade by Prucha (1915), Temple (1916), Wilson (1917), Fellers (1918), Barthel (1921), Glöbel (1926), Fred, Whiting and Hastings (1926) and many others have added much information to all phases of the subject. References having a direct bearing on the experimental data to be presented in this paper will be discussed in the following historical review. The discussion can best be classified under the headings: Nitrogen Fixation, Commercial Cultures, and Methods of Culture Testing.

Nitrogen Fixation

Stoklasa (1895) and Frank (1904) were the first investigators to present theories concerning the seat of nitrogen fixation in leguminous plants. They concluded that nitrogen is fixed in the leaves of the plants and that bacteria in the root nodules probably play an accessory role in the process. The work of Kossowitch (1892), subsequently verified by

Nobbe and Hiltner (1899), Golding (1904), Herke (1913) and Whiting (1915) shows that nitrogen fixation does not take place in the leaves of the plants, but is confined to the roots. The work of Gerretson, Gryns, Sach and Sohngen (1924) suggests that a bacteriophage is present in the plants which possesses the power of breaking down, or rather dissolving, the bacteria in the root nodules. In this state their contents are available for the plants.

The amount of nitrogen actually fixed by different inoculated legume plants varies. The details of this phenomena are shown in the reports of the following investigators.

Investigator	Legume Inoculated	Nitrogen Fixed Mgms. per Plant
Hopkins (1904)	Cowpeas	12.9
Alway & Pickney (1910)	Alfalfa	4.1
	Clover	120-250
Wright (1925)	Soybeans	400

Giobel (1926) also presents (in table form) results showing the amounts of nitrogen fixed by field legumes in various countries of the world.

Warrington (1891), Lipman, Owen, Blair, McLean (1913), Lyon and Bizzell (1913), Albrect (1921), Brown and Stallings (1921), and Fred, Wright and Frazier (1921) have all contributed information demonstrating that the growth of legumes on unproductive soils tends to increase their productivity by the addition of soil nitrogen. Lyon, Bizzell and their associates (1924), after several years of intensive study and investigation, find that the growth of legumes results in a greater accumulation of

of nitrate-nitrogen in the soil.

Gilbert (1889), Alway and Bishop (1912), and Fred and Graul (1919) present experimental data to show that more nitrogen is taken from the soil by the plants than is fixed by the bacteria from the atmosphere. Fred and Graul also report that 40.3% of the total nitrogen in Alfalfa crops comes from the air and 59.7% from the soil.

Lipman and Blair (1913) conclude that the number of nodules on the roots of the plants is a true index of the nitrogen fixing ability of the bacteria. Fred (1921) supports this claim and further states that the size and location of the nodules are also important and closely related to the activity of the bacteria. He concludes that a small number of large nodules indicates more nitrogen fixation than a large number of small nodules. Nodules located near the surface of the soil on the main roots fix the most nitrogen while those located on the tap root, a considerable distance from the surface, fix little or no nitrogen.

Commercial Cultures

A German laboratory was the first to place on the American market pure (?) cultures of Rhizobium leguminosarum. These cultures were introduced in 1896 and sold under the trade name "Nitragin". The bacteria were contained in a fine humus soil which was spread upon and mixed with the seed immediately before planting. Early reports of these cultures are very unsatisfactory. In 1902 the United States Department of Agriculture

undertook the preparation and sale of cultures. Narrow strips of cotton were inoculated by immersion in a broth culture medium containing pure cultures of Rhizobium leguminosarum. The inoculated cotton was dried and shipped to the farmer with a bottle of sterile liquid culture medium. Upon receipt, the farmer placed the dried cotton in the sterile medium and allowed it to remain for at least three days. The newly inoculated medium was mixed with the seed and the latter immediately planted. These cultures proved unsatisfactory and their manufacture was soon discontinued.

Harrison, in 1905, devised a nitrogen-free culture medium which solved many of the difficulties of earlier culture production. The new medium supplied a satisfactory substrate for maintaining growth and viability of the bacteria between preparation in the laboratory and use in the field. Between the years 1903 and 1910 the following American laboratories began the preparation and sale of bacteriological cultures for the inoculation of legume seed:

The H. K. Mulford Co.	1903
The Albert Dickinson Co.	1904
The Harp-Thomas Co.	1906
Seed & Soil Co. of N. J.	1906
Armour Fertilizer Co.	1907
Nitro Germ Co. of Savannah	1909
Alphano Humus Co.	1909
Standard Seed & Soil Inoculation Co.	1910
H. M. Scott Co.	1910

Since then competition and inability to produce satisfactory cultures have forced some of these laboratories from the market. Many State

Experiment Stations are now producing and distributing cultures. The H. K. Mulford Co., The Albert Dickinson Co. and the Earp-Thomas Co. dominate the Massachusetts' Market and at least two other companies are selling a few cultures in this state.

Methods of Culture Testing

The first records concerning the quality of commercially prepared legume cultures did not come from the laboratories, but from the farmers who made practical use of them in the field. Farmers using "Nitragin" were so emphatic in their denunciations of these early cultures that the United States Department of Agriculture began an extensive investigation of culture quality. The results of this study were published by G. T. Moore, (1902) who concludes that:

"1. The method of transferring soil is objectionable.

2. "Nitragin" is not successful.

3. The organisms must be cultivated in a nitrogen-free medium."

Hall, Harding and Prucha (1905) conducted approximately 40 examinations of various commercial cultures. They conclude that "while the packages are satisfactory the methods of applying the cultures are entirely unsatisfactory in all cases."

Ball (1906) tested several cultures, including "Nitro-cultures", sold by the Nitro Germ Company of Savannah, and concluded that in no case was artificial inoculation as successful as natural inoculation.

Lewis and Nicholson (1905) examined several "Nitro-cultures" and found that although bacteria were present in very small numbers, they were pure strains of another organism not associated with nitrogen fixation in legumes.

Harding and Prucha (1905) make the following terse statements regarding results of their examinations of commercial cultures:

"Packages cost $2.00. The cost of production is 10¢. The packages are worthless."

Lipman (1910) reports satisfactory results with "Nitragin" and "Farmogerm" (Earp-Thomas Co.) cultures.

Noyes and Blair (1918) report satisfactory results with commercial cultures, although they recommend larger cultures for the best results.

Fellers (1918) reporting the first annual tests on commercial cultures sold in New Jersey finds that 40% of the 49 cultures tested were either "very poor" or "worthless". Since 1918 the New Jersey Agricultural Experiment Station has conducted annual control tests of the cultures sold in that state. A summary of the results for the years 1918-1928 shows a total of more than 800 cultures officially tested and approximately 43% were reported as either "poor" or "unsatisfactory".

Erdman and Wilkins (1928), reporting studies of commercial cultures for inoculating soybean seed, conclude: "Home cultures are better adapted to one variety of soybeans than to others. Cultures vary widely in

inoculating efficiency. Those from the factory were apt to be more efficient than the same brand from a dealer, although this superiority was not apparent in 1926."

The methods employed for testing commercial legume cultures have varied little since their inception. Slight changes in technique have been made, but in general the methods used in 1905 and today are similar. Cultures are subjected to plating experiments in order to determine the number of viable nitrogen-fixing bacteria and possible contaminants. Seed is inoculated, planted and grown under controlled conditions in a low nitrogen content soil in order to estimate the nodulating power (infectiveness) of the cultures. At the end of a prearranged period of growth (usually 30 days) the plants are examined for their general health and the average number of nodules per plant. The nitrogen fixing abilities of the cultures are entirely based on these observations and the culture producing the highest average number of nodules is judged the most active. The theoretical consideration involved in these examinations presupposes a direct relationship between the number of nodules and the amount of nitrogen fixed by the bacteria. This assumption is not always correct in the light of experimental knowledge of strain variation among these bacteria. The studies here presented offer a new method for examining commercial cultures which precludes differences in the nitrogen-fixing powers of the organisms. Cultures of symbiotic nitrogen-fixing bacteria are used to increase plant and soil nitrogen, not to grow nodules on the roots of the legume

plants. Therefore, the value of a culture must be judged by an actual measurement of the nitrogen it will fix in the plants.

The new method, proposed in this study, also allows a grading of the cultures of intermediate quality. This cannot be done accurately by the methods now available.

Media and Methods

Plating Experiments: Standard methods of plating were employed to determine the relative numbers of viable nitrogen-fixing bacteria in the cultures and to study their purity.

The following medium was used for isolating the legume bacteria:

$CaCO_3$	5.0 grams
KH_2PO_4	0.2 "
NaCl	0.2 "
$MgSO_4$	0.2 "
$CaSO_4$	0.5 "
Mannite	15.0 "
Agar	12.0 "
Distilled water	1000 CC

The reaction was adjusted to pH 7.0, and the medium sterilized in the autoclave at 15 lbs. pressure for 20 minutes.

Standard nutrient agar ("Standard Methods of Water Analysis, 6th ed., 1925") was employed in the cultivation of non-nitrogen-fixing bacteria.

Czapek's medium was selected for the isolation of contaminating molds. It's composition follows:

Cane Sugar	30.0 grams
$NaNO_3$	2.0 "
$MgSO_4\ 7H_2O$	0.5 "
KH_2PO_4	1.0 "
KCl	0.5 "
$FeSO_4$	0.01 "
Agar	12.0 "
Distilled water	1000 CC

The material was sterilized in the autoclave at 15 lbs. pressure for 20 minutes.

The plating technique follows the method described in "Standard Methods of Milk Analysis, 5 ed., 1923.", except that ten c.c. amounts

of inoculum were used in all cases instead of one c.c. amounts.

The cultures were prepared for plating by filling the bottles to the shoulder with a measured amount of sterile water and thoroughly shaking to insure an even suspension of the bacterial cells. Dilutions were prepared from the suspension and plated in triplicate on Ashby's medium, standard nutrient agar and Czapek's medium. An incubation temperature of 30° C. for at least 72 hours was used in all experiments. Final results express the average bacterial count on the plates multiplied by the amount of water added to the culture bottle in the preparation of the original suspension.

Tumblers and sand: Ordinary household jelly tumblers (8 oz.), 6½ inches in length, 2½ inches top diameter and tapering to 1¾ inches at the bottom were used for plant culture.

The sand used in all experiments contains an extremely small amount of nitrogen (0.00023%). Two hundred grams were placed in each tumbler and the sides of the tumblers wrapped with heavy paper to exclude light from the plant roots. Prior to sterilization in the autoclave at 15 lbs. pressure for at least 6 hours, temporary paper caps were placed on the tumblers to prevent subsequent air contamination. After sterilization the sand was placed in a dry, dust-free room for at least seven days before use to eliminate any possible toxic effect upon the plants.

Seed: Although the same strains of alfalfa and red clover seed were

used in all experiments only small portions were sterilized for immediate use. The seeds to be sterilized were wrapped in cheese cloth and completely submerged in a 1-1,000 solution of silver nitrate for 10 minutes. After the exposure the bag was removed, drained and finally washed with sterile water until all traces of silver nitrate solution had disappeared.

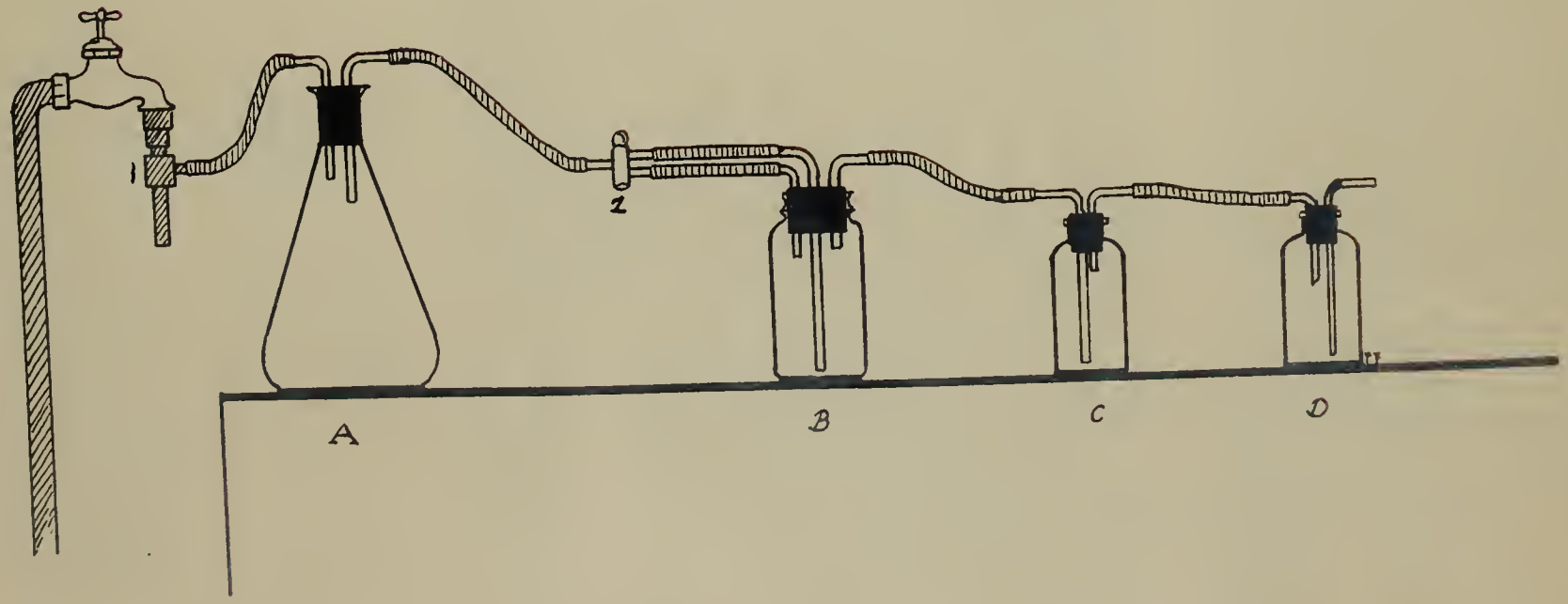
Most investigators transfer the seed to at least four successive beakers containing sterile water to remove all traces of disinfectant. This method increases the danger of contamination and requires a large amount of equipment. The writer has devised a method which is an improvement upon the above mentioned technique. A sketch of the apparatus is shown in Figure 1.

Sterile seed are placed in an empty wide mouthed sterile bottle (B). By means of an aspirator (1) and a two way stop-cock (2) water is drawn from Bottle (C) into Bottle (B). The seeds are allowed to remain in the water for 2 - 4 minutes and then the water is withdrawn into catch-bottle (A). This procedure is repeated at least four times in order to remove all traces of silver nitrate solution. Bottle (D) contains about 30 C.C. of sulphuric acid which serves to remove contaminating organisms from the air before it reaches the sterile washing bottle. The apparatus was found more efficient for seed handling than successive washing in beakers, and the results were entirely satisfactory.

The sterile, washed seed are placed in sterile Petri dishes con-

FIGURE I

APPARATUS FOR WASHING SEED



taining several layers of sterile filter paper. The Petri dishes are then placed in the 37°C incubator until the seeds are dry.

Before planting the seeds are germinated by placing them in a Petri dish containing sterile moist filter paper for 48 hours at room temperature.

Twenty germinated seeds are individually selected with flamed forceps and planted in each tumbler. Ungerminated seeds were always discarded as a possible means of adding nitrogen to the experimental sand. After planting the optimum moisture content (15-18%) was adjusted.

Instead of inoculating the seed just before planting with an aliquot portion of the culture based on the amount of seed, the writer adopted the following technique which permitted a heavier and more even inoculation. One c.c. of the original suspension of the culture was added to 20 c.c. of sterile water and thoroughly shaken. One c.c. of the 1-20 dilution was pipetted onto the sand of each tumbler.

Immediately after adding the inoculum and every 15 days during the growth period each tumbler received 10 c.c. of the following soil nutrient solution:

KCl	10.0 grams
$Ca\ SO_4$	2.5 "
$Mg.\ SO_4$	2.5 "
$Ca_3\ (PO_4)_2$	2.5 "
$Fe\ PO_4$	2.5 "
$K_3\ PO_4$	2.5 - 3.5 grams

The salts are ground together in a mortar. Fifteen grams of the mixture are added to one litre of distilled water. After mixing the resulting suspension stands over night in a tall cylinder. The clear supernatent liquid is decanted and sterilized in the autoclave at 15 lbs. pressure for 20 minutes.

Preliminary experiments were conducted to study the rate of nitrogen increases in the plants during the Spring, Summer and Fall. The plants were first placed in the top floor of the Bacteriology building and received sunlight and fresh air by means of a large glass skylight. The tumblers were examined daily and moisture content adjusted. No attempt was made to protect the tumblers from air contamination, consequently daily examinations were made for algae, moulds and insects. Tumblers showing the presence of contaminants were immediately discarded.

In the next experiment, the plants were grown under greenhouse conditions at the East Experiment Station during the late Fall and early Winter. A temperature of approximately 25 C. was maintained throughout the experiment. Methods for controlling the moisture content of the sand and search for contamination were unchanged.

Methods for Determining Nitrogen: At the end of the growth period the plants were removed to the laboratory and washed free from sand by a gentle stream of water. The total number of plants representing one culture, or, as in the case of the preliminary experiments, the total number of plants representing one growth period, were placed in a wide shallow dish containing water. The number of plants for a single nitrogen determination (10) were selected and the number of nodules on each plant recorded. The selected plants were placed in a long necked 800 C.C. capacity Kjeldahl flask, to which was added 2 grams of potassium sulphate, 5 c.c. of a 5% copper sulphate solution, 50 c.c. of nitrogen-free water and 20 c.c. of

concentrated nitrogen-free sulphuric acid (C.P.). After complete digestion of the plant material the flask was cooled and 200 c.c. of ammonia-free water and a pinch of talcum added to the digested material. Seventy c.c. of a 50% solution of sodium hydroxide were carefully introduced and the flask immediately connected to the distillation apparatus.

Two hundred c.c. of distillate were collected in a 250 c.c. volumetric flask containing 50 c.c. of ammonia-free water. After thorough mixing a definite amount (1-5 c.c.) was transferred to each of two 50 c.c. Nessler tubes. These tubes were filled to the mark with ammonia-free water and inverted several times to insure thorough mixing of the contents. One c.c. of Nessler's reagent was added to each tube and after 5 minutes, comparison was made with freshly prepared standards. The involved technique and the preparation of the standard ammonia solution are described in "Standard Methods of Water Analysis", 5th edition, pages 13 and 14. Total nitrogen in the plants can be directly calculated by comparing unknown tubes with standards containing definite quantities of nitrogen.

Control Tests: All media and apparatus used in the plating experiments were regularly tested for sterility. Water and soil nutrient solution were tested for the presence of nitrogen.

All equipment and apparatus used in the nitrogen studies were chemically cleaned with an acid solution of potassium dichromate.

In all experiments uninoculated seed were planted to insure that there had been no introduction of extraneous nitrogen.

After sterilization and washing the seed were again tested for

sterility.

At the conclusion of each experiment several gram samples of tumbler sand were selected at random and submitted to bacteriological examinations for the presence of non-symbiotic nitrogen-fixing micro-organisms.

Uninoculated plants showing nodule development were never employed in nitrogen studies on the control plants.

EXPERIMENTAL

Before beginning a detailed discussion of the experimental work, it appears advisable to present a brief plan of attack for this particular problem. It is hoped that the final results will be of such a nature that the methods and technique can be adopted as a new routine laboratory method for testing the quality of commercial legume cultures. In all cases attempts will be made to simplify and standardize technique. Economy of time and equipment must also be considered at all times.

Information concerning the total numbers of nitrogen fixing bacteria can be obtained by means of standard plating technique. Data concerning the possible contamination of the cultures thru poor laboratory procedure can be made available by employing three types of culture media, each especially adapted to the cultivation of certain types of microorganisms. The writer is forced to accept the standard plating methods for determining total numbers of bacteria, although appreciating its inaccuracies. Research may some day give us a better technique, but the question is debatable. Results, although only approximate, give an estimate of existing conditions. No effort is made in this investigation to correlate numbers of organisms present in the cultures with experiments on nitrogen fixation in the plants. The presence of large numbers of organisms cannot be assumed as a true index of culture quality.

In order to measure nitrogen fixability, experimental conditions can be controlled to exclude nitrogen from sources other than the seed and

bacteria. The small amounts of available nitrogen found in these studies make it necessary to substitute quantitative colorimetric comparison determinations for quantitative titration methods in the final nitrogen studies. Adapting colorimetric methods increases accuracy to 0.002 mgm. of nitrogen. Total nitrogen cannot be determined accurately in single plants and preliminary experiments must be conducted to determine the optimum number of plants for a single determination. A study of the relationship of growth and increased nitrogen in the plants must be made to insure accurate quantitative differences between the fixing power of strains and cultures. When this information is available a definite study of the relative nitrogen fixing power of the cultures can be made. It appears advisable to duplicate the nitrogen studies in order to ascertain the effect of seasons upon infectibility and nitrogen fixation.

Preliminary Experiments

Plating Experiments: The study includes at least four cultures each of clover and alfalfa prepared and sold by the H. K. Mulford Company, The Albert Dickinson Company and the Earp-Thomas Company. The cultures were purchased in several retail stores throughout the state and thus represent the quality of products supplied to the agriculturist. A total of 32 cultures were examined for viability and contamination according to the methods described in the section "Media and Methods." The results of these experiments are shown in Table 1.

TABLE 1

RESULTS OF PLATING EXPERIMENTS

Lab. No.	Type of Inoculum	Manufacturer	Trade Name	Size of bottle	Total Number of R. leguminosarum	Contaminating Organisms
60	Alfalfa	Mulford Co.	Mulford Cultures	1 bu.	7,200,000,000	None
61	"	" "	" "	" "	8,000,000,000	None
62	"	" "	" "	" "	8,400,000,000	None
82	"	" "	" "	" "	200,000,000	None
83	"	" "	" "	" "	160,000,000	None
63	Clover	" "	" "	" "	24,000,000	None
64	"	" "	" "	" "	12,000	None
65	"	" "	" "	" "	40,000	None
78	"	" "	" "	" "	None	None
84	"	" "	" "	" "	None	None
66	Alfalfa	Albert Dickinson	Nodogen	" "	5,600,000,000	None
67	"	" "	"	" "	7,500,000,000	None
68	"	" "	"	" "	3,000,000,000	None
85	"	" "	"	" "	1,250,000,000	None
69	Clover	" "	"	" "	5,000,000,000	None
70	"	" "	"	" "	1,200,000,000	None
71	"	" "	"	" "	2,500,000,000	None
86	"	" "	"	" "	1,350,000,000	None
87	"	" "	"	" "	1,000,000,000	None
72	Alfalfa	Earp-Thomas	Farmogerm	" "	48,000,000	None
73	"	" "	"	" "	18,000,000	None
74	"	" "	"	" "	24,000,000	None
88	"	" "	"	" "	600,000,000	None
89	"	" "	"	" "	750,000,000	Present
90	"	" "	"	" "	900,000,000	None
91	"	" "	"	" "	600,000	None
75	Clover	" "	"	" "	99,000,000	Present
76	"	" "	"	" "	177,000,000	None
77	"	" "	"	" "	400,000,000	None
79	Alfalfa	" "	Humogerm	" "	950,000,000	Present
80	Clover	" "	"	" "	200,000,000	None
81	"	" "	"	" "	None	None

This table shows that three of the cultures contained no viable symbiotic nitrogen fixing bacteria. They must be classified as worthless. Three other cultures revealed the presence of contaminating organisms. The extent of contamination, however, was limited, and the total number of viable nitrogen fixing bacteria compared favorably with the best cultures. These cultures, although contaminated, cannot be classed as worthless.

It is interesting to note the wide variations in numbers of bacteria present in the cultures. Cultures prepared by the Albert Dickinson Company show the highest average bacterial count. Nine of these cultures were examined and in every case the total count was over one billion viable nitrogen-fixing bacteria per culture. The Mulford cultures contained the lowest averagebacterial count. Their cultures for alfalfa contained large numbers of viable bacteria, but the clover cultures were extremely low in numbers of bacteria. Two of the clover cultures contained no bacteria, and in two others there were considerably less than 100,000 organisms. The remaining clover culture contained 24 million bacteria, which the writer would consider a satisfactory bacterial content. The Earp-Thomas cultures, in every case, contained less than one billion organisms, but, with the exception of two cultures, well over a million. Of these two exceptions--both "Humogerm" clover cultures--one contained absolutely no viable bacteria, the other contained 600,000, which is unsatisfactory.

Fellers (1918), in a report of the examination of commercial legume cultures sold in New Jersey in that year, states that when less than one nodule per plant was produced, the cultures were classified as very poor. Theoretically, to produce one nodule per plant, there must be at least one bacterial cell for each seed. Better results are doubtless obtained if several organisms, instead of one, are attached to a seed. Fellers estimated the average number of seeds of our common legumes usually planted per acre. For alfalfa the number is 5,100,000, and for red clover 3,300,000. Therefore, to produce minimum inoculation of the seed there must be at least 5,100,000 viable symbiotic nitrogen-fixing bacteria present in a one-acre size alfalfa culture. Similarly, there must be at least 3,300,000 viable organisms present in a one-acre size clover culture.

An examination of Table 1 indicates that a total of six cultures do not contain sufficient bacteria to supply minimum inoculation of the seed. Three of these are cultures already mentioned as containing no viable nitrogen-fixing bacteria. Of the remaining three cultures, two are Mulford clover cultures and the other an Earp-Thomas "Farmogerm" culture. One of the Mulford products contained only 40,000 bacteria and the other but 12,000. There is little doubt that these cultures are unable to produce a minimum inoculation of the seed, and consequently can be classified as worthless. It is worthwhile to note that five Mulford clover cultures were examined, four of which were of little or

no value. If these results represent a cross section of the quality of the total clover cultures produced by this laboratory in 1927, protection should be given the purchaser by regulatory measures.

The culture remaining in the group showing a bacterial content insufficient for minimum inoculation was an Earp-Thomas clover culture. On the whole, the Earp-Thomas cultures contained large numbers of viable nitrogen-fixing bacteria. The Albert Dickinson "Nod-o-gen" cultures contained sufficient bacteria for very heavy inoculation of the seed. However, in most cases there were enough cells for an average of more than 100 per seed.

In summarizing the results of plating experiments with commercial cultures sold in Massachusetts it can be said from the evidence at hand that the Albert Dickinson Company and the Earp-Thomas Company are producing cultures of very pure quality and containing sufficient viable cells to produce a heavy inoculation of the seed. On the other hand, the H. K. Mulford Company is distributing cultures of questionable quality.

Nitrogen Studies: Preliminary experiments were conducted to determine whether the methods and technique were workable and efficient, to determine the shortest time of growth necessary to show possible variations in individual strains of bacteria, and to determine whether or not experimental conditions can be controlled to exclude all nitrogen, except that present in the seed and fixed by the bacteria.

The first experiment determines the average nitrogen content of 10 alfalfa and 10 clover seeds. A series of 50 nitrogen determinations, made in five groups of 10 determinations each, were conducted on each type of seed. The results of the determinations are shown in Table 2 and Plate 1. Table 2 gives the results in mgms. of nitrogen per 10 seeds. The average nitrogen content of 10 alfalfa seeds is 1.20 mgms. Table 3 and Plate 1 gives similar results with red clover seed. The average nitrogen content of 10 seeds is 0.90 mgm.

TABLE 2

Average Nitrogen Content by Group
Determination on Red Clover Seeds.

First Group		Second Group		Third Group	
Test No.	Mgm.Nitrogen in Seed	Test No.	Mgm.Nitrogen in Seed	Test No.	Mgm.Nitrogen in Seed
1	0.97	1	1.07	1	0.83
2	1.07	2	0.87	2	0.87
3	1.07	3	0.87	3	0.87
4	0.95	4	0.97	4	0.77
5	1.02	5	0.87	5	0.77
6	0.80	6	0.87	6	0.77
7	0.87	7	0.94	7	0.77
8	1.10	8	0.81	8	0.80
9	1.00	9	0.97	9	0.80
10	0.90	10	0.88	10	1.00
Average	0.97	Average	0.91	Average	0.82

Fourth Group		Fifth Group	
Test No.	Mgm.Nitrogen in Seed	Test No.	Mgm.Nitrogen in Seed
1	0.85	1	0.95
2	0.78	2	1.10
3	0.87	3	1.07
4	0.99	4	0.86
5	0.93	5	0.83
6	0.90	6	0.91
7	0.89	7	0.95
8	1.02	8	0.87
9	1.05	9	0.81
10	0.98	10	0.75
Average	0.92	Average	0.91

TABLE 3

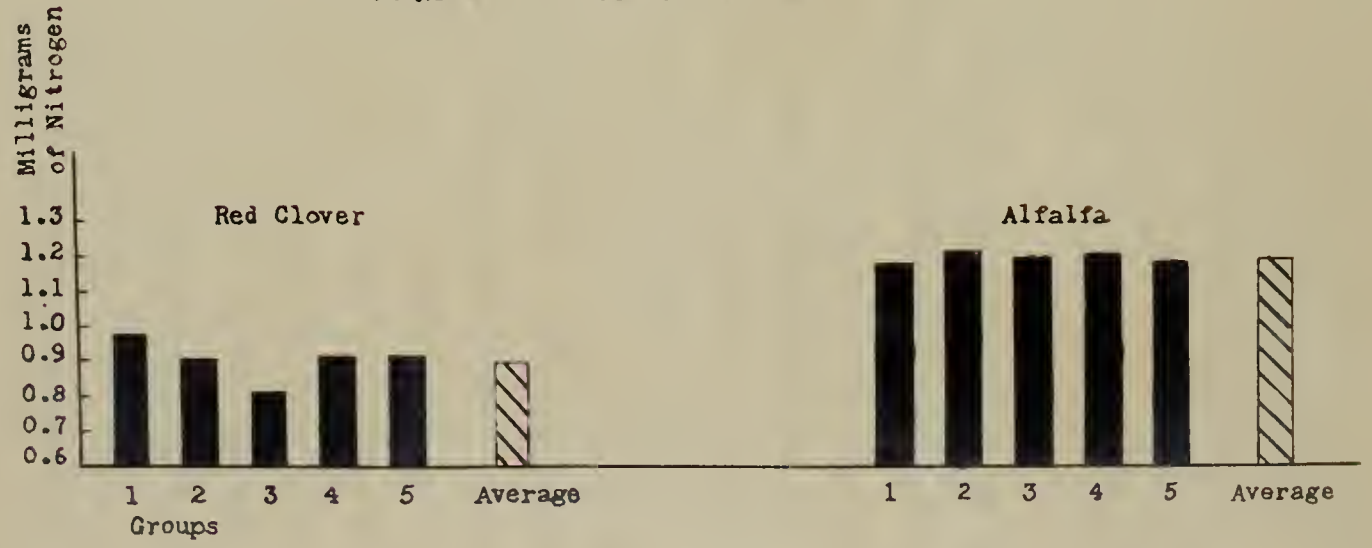
Average Nitrogen Content by Group Determination in Alfalfa Seeds.

First Group		Second Group		Third Group	
Test No.	Mgm. Nitrogen in Seed	Test No.	Mgm. Nitrogen in Seed	Test No.	Mgm. Nitrogen in Seed
1	1.16	1	1.31	1	1.30
2	1.16	2	1.18	2	1.21
3	1.18	3	1.30	3	1.18
4	1.11	4	1.17	4	1.18
5	1.19	5	1.19	5	1.18
6	1.22	6	1.25	6	1.22
7	1.31	7	1.22	7	1.27
8	1.18	8	1.30	8	1.20
9	1.21	9	1.22	9	1.11
10	1.18	10	1.17	10	1.15
Average	1.17	Average	1.23	Average	1.20

Fourth Group		Fifth Group	
Test No.	Mgm. Nitrogen in Seed	Test No.	Mgm. Nitrogen in Seed
1	1.11	1	1.31
2	1.18	2	1.11
3	1.20	3	1.17
4	1.19	4	1.19
5	1.31	5	1.22
6	1.24	6	1.20
7	1.27	7	1.21
8	1.18	8	1.25
9	1.21	9	1.11
10	1.22	10	1.17
Average	1.21	Average	1.19

PLATE 1

Graphic Representation of Average Nitrogen Content by Group Determination on Alfalfa and Red Clover Seeds.



The second preliminary experiment determines the average nitrogen content of the sand to be used for growing legume plants in the laboratory. Ten-gram samples of the sand were selected and their total nitrogen content determined by the Kjeldahl technique. The results are given in Plate 2. The average nitrogen content of 10 grams of the sand is 0.023 Milligram, or 0.00023%.

Another series of preliminary experiments studied the effect of time upon nitrogen fixation in the legume plant grown under experimentally controlled conditions. Red clover seed were inoculated with the Experiment Station strain No. 100, of Rhizobium leguminosarum and the plants grown under the conditions outlined in the section "Media and Methods". Uninoculated seed were also planted and grown at the same time and under the same conditions. At the end of 7, 10, 12, 15, 20, 25, 30, 40 and 60 days of growth nitrogen determinations were made upon the inoculated plants. Similarly, the uninoculated control plants were tested for total nitrogen after 20 days of growth. The results of the experiment are given in Tables 4 and 4a and Plate 3. Table 4 presents the results of the individual tests at the end of each growth period. The results are expressed in the average number of nodules and the total mgms. of nitrogen.

Single variations appear in the determinations noted in this table, but totally within the experimental error of the method.

Table 4a presents the results of nitrogen determinations upon the uninoculated plants.

PLATE 2

Results of Nitrogen Determinations on Sand

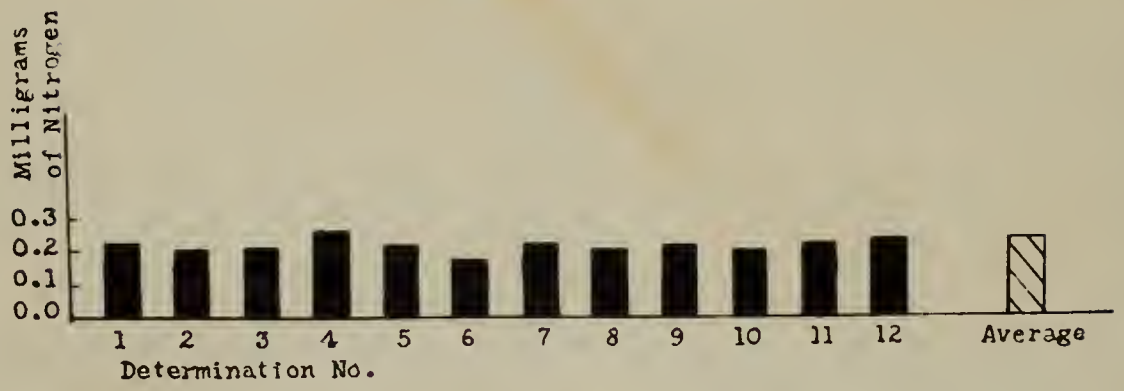


TABLE 4

Nodulation and Nitrogen Determinations on Inoculated Red Clover Plants at the End of Different Growth Periods.

7 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	0	0.88
2	0	0.84
3	0	0.96
4	0	0.92
5	0	1.00
6	0	0.82
7	0	0.82
8	0	1.00
9	0	0.98
10	0	0.84
11	0	1.03
12	0	0.96
Average	0	0.92

10 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	0	0.81
2	0	0.89
3	0	1.00
4	0	0.83
5	0	0.91
6	0	0.96
7	0	1.03
8	0	0.94
9	0	0.91
10	0	0.80
11	0	0.88
12	0	1.01
Average	0	0.91

12 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	0	0.93
2	0	0.92
3	0	0.91
4	0	0.90
5	0	0.88
6	0	0.87
7	0	0.92
8	0	0.88
9	0	0.89
10	0	1.00
Average	0	0.91

15 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	0.6	0.89
2	1.1	0.80
3	2.2	0.76
4	0.9	0.91
5	1.1	0.90
6	1.3	0.79
7	0.7	0.90
8	0.7	0.83
9	0.7	0.84
10	0.6	1.02
11	0.1	1.06
12	0.3	0.80
13	1.4	0.89
14	2.1	0.88
15	2.4	0.95
16	2.0	0.96
17	1.8	0.90
18	2.1	0.98
19	2.1	0.98
20	2.8	1.00
21	3.0	1.00
22	2.2	0.99
23	2.9	1.01
24	3.0	0.94
Average	1.5	0.91

TABLE 4 (Cont.)

Nodulation and Nitrogen Determinations on Inoculated Red Clover Plants at the End of Different Growth Periods.

20 Days

Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	2.8	0.92
2	2.8	0.88
3	2.6	0.85
4	2.8	0.82
5	2.9	0.98
6	3.0	0.82
7	2.9	0.91
8	2.9	0.93
9	2.7	0.98
10	3.0	0.92
11	2.8	0.84
Average	2.8	0.89

25 Days

Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	2.8	0.84
2	3.1	1.15
3	3.0	1.10
4	3.2	1.00
5	3.0	0.92
6	3.4	0.96
7	2.9	0.88
8	3.5	1.02
9	3.2	0.92
10	3.4	0.98
11	3.4	1.01
12	2.1	1.30
13	1.8	1.22
14	1.5	1.00
15	0.9	1.15
16	2.0	1.15
17	2.7	1.20
18	0.9	1.05
19	1.1	1.06
20	1.8	1.06
21	1.9	1.12
22	1.2	0.95
23	1.6	0.95
Average	2.3	1.04

30 Days

Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	4.7	1.06
2	4.6	1.26
3	4.9	1.30
4	4.9	1.24
5	5.2	1.23
6	5.0	1.22
7	4.8	1.20
8	4.8	1.23
9	5.3	1.47
10	4.9	1.20
11	4.7	1.03
12	5.4	1.33
Average	4.9	1.23

TABLE 4 (Cont.)

Nodulation and Nitrogen Determinations on Inoculated Red Clover Plants at the End of Different Growth Periods.

40 Days			60 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants	Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	7.2	1.15	1	5.3	2.00
2	8.1	1.74	2	8.0	2.37
3	8.0	1.65	3	11.8	2.01
4	8.5	2.19	4	8.7	2.81
5	8.0	1.76	5	15.5	2.90
6	8.3	2.19	6	9.3	2.87
7	7.9	1.76	7	6.1	2.90
8	7.0	1.59	8	5.1	3.18
9	7.2	1.62	9	5.6	2.79
10	8.0	2.24	10	7.2	2.35
11	9.0	2.38	11	5.3	2.65
12	8.3	1.50	12	6.9	3.15
13		1.75	Average	7.8	2.68
14		2.00			
15		2.00			
16		2.01			
17		2.18			
18		2.06			
19		1.52			
20		1.40			
21		1.47			
Average	7.9	1.83			

TABLE 4a

Nitrogen Determinations on Uninoculated Red Clover Plants at the End of Different Growth Periods.

20 Days	
Test No.	Mgms.Nitrogen in Plants
1	0.86
2	0.91
3	0.84
4	0.82
5	0.90
6	0.87
7	0.94
8	0.91
9	0.90
10	0.93
11	0.87
12	0.88
Average	0.89

25 Days	
Test No.	Mgms.Nitrogen in Plants
1	0.97
2	1.00
3	0.81
4	0.94
5	0.91
6	0.89
7	0.98
8	1.10
9	1.05
10	1.00
11	0.87
12	0.97
Average	0.96

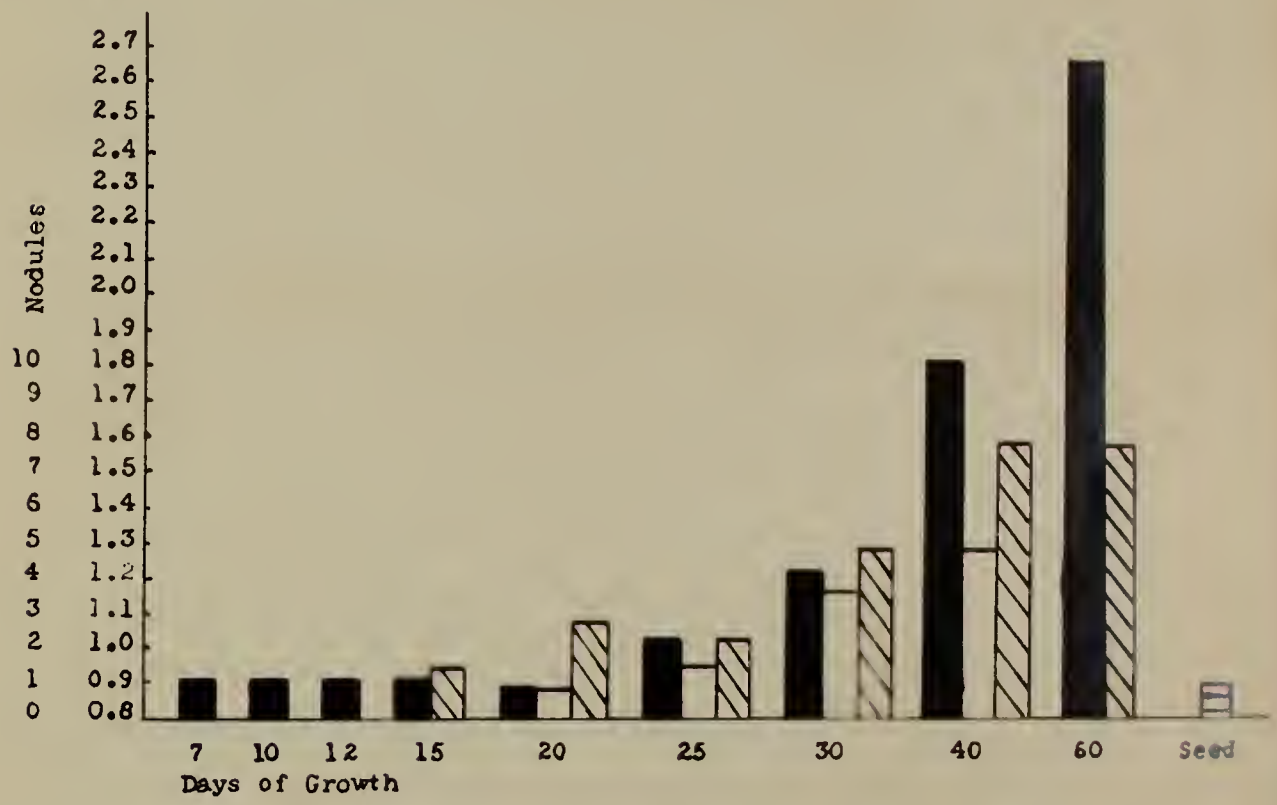
30 Days	
Test No.	Mgms.Nitrogen in Plants
1	0.98
2	1.15
3	1.23
4	1.15
5	1.26
6	1.17
7	1.15
8	1.18
9	1.31
10	1.27
11	1.07
12	1.00
Average	1.17

40 Days	
Test No.	Mgms.Nitrogen in Plants
1	1.27
2	1.34
3	1.35
4	1.29
5	1.37
6	1.25
7	1.10
8	1.45
9	1.32
10	1.22
11	1.31
12	1.30
Average	1.29

Plate 3 presents a better picture for summarizing the results of this experiment. It indicates the averaged results of the individual tests at the end of each period of growth. The solid columns represent the average amount of nitrogen in the inoculated plants. The outlined columns represent the average amount of nitrogen in the uninoculated plants. The cross-hatched columns represent the average number of nodules.

PLATE 3

Average of All Nitrogen Determinations and Nodulation on Red Clover Plants at the End of Different Growth Periods



The results shown by table and graph prove the workability of the methods and technique. The procedure employed in the quantitative measurement of nitrogen are sufficiently delicate to show variations of 0.001 - 0.002 milligram. The method can be used to determine the nitrogen fixing powers of various strains of bacteria, and to classify the inoculating value of cultures.

Waksmen (1927) and Greaves (1922) report that increases in the nitrogen content of inoculated legume plants occur within the first 12 to 15 days of growth. They do not, however, present experimental data to support their conclusions, nor has the writer been able to find any information in the literature which would justify these conclusions. The above experiment clearly demonstrates the time required for the conversion of fixed nitrogen into a form available for plant development, and the rate of increase in the nitrogen content of inoculated plants throughout their early growth. The same graph shows that there was no measurable increase in the nitrogen content of red clover plants until between the 20th and 25th days of growth. Beyond this period the increase was rapid nearly doubling itself every 10 days.

Many investigators have reported that nodules are first observed on the roots of the plants between the 12th and 15th days of growth. The results of the writer confirm this statement. Although nodules are present on the plants after 15 days, nitrogen increase does not take place until after 20 days. Nodulation surely is not indicative of nitrogen fixation at this time. The absence of any nitrogen increase possibly indicates

that fixation does not commence until after 20 days of plant growth. A definite explanation requires further research on this phase of the subject.

The uninoculated plants obtained nitrogen from some source which is unknown. However, every possible precaution had been taken to exclude nitrogen other than that in the seed and that fixed by the bacteria. Nodules were not present on the plants, which indicates that nitrogen must have come either from the sand, the water, or the soil nutrient solution. The water and nutrient solution were carefully checked for nitrogen and none was found, therefore they can be eliminated. The sand remains as the only possible source. Total nitrogen determinations of the sand show an average of 0.00023% nitrogen and this amount appears too small to seriously affect results. All attempts to remove any of this nitrogen by washing the sand in acid has resulted in failure. Since each tumbler contained 200 grams of sand, it can be estimated that there was 0.46 mgm. of nitrogen in the sand of each tumbler. The only apparent explanation for an increase in the nitrogen content of the uninoculated plants is that some of the sand nitrogen may have been utilized by the plants. An attempt was made to verify this point by nitrogen determinations on the sand before and after plant growth. The individual variations between samples from the same tumbler varied to such an extent that it was impossible to show any loss due to plant absorption. However, after 40 days of growth the uninoculated plants show an increase of approximately 0.50 milligram

of nitrogen. It was estimated that the average nitrogen content of the sand in the tumblers was 0.46 mgm. If the plants remove 0.50 milligram there should be no nitrogen left in the sand at the end of the growth period. This was not the case; the sand contained approximately the same amount at the end of the growth period that it did at the beginning. The explanation, then, for this increase is not apparent, unless one wishes to dispute plant physiologists by saying that these plants fixed nitrogen directly from the atmosphere. The writer is not prepared at present to make this statement. It was finally decided to deduct the average increase in nitrogen in the uninoculated plants from that in the inoculated plants in all cases and thus leave a net gain due to bacterial activity.

The above experiment was repeated using alfalfa seed and the Experiment Station strain 101 of Rhizobium leguminosarum; the object being in this experiment to study a possible similarity between the rate of nitrogen increase in clover and alfalfa plants.

Table 5 gives the results of the individual tests of the inoculated plants for each growth period. Table 5a presents the results of the nitrogen determinations on the uninoculated plants.

TABLE 5

Nodulation and Nitrogen Determinations on Inoculated Alfalfa Plants at the End of Different Growth Periods.

7 Days			10 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants	Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	0	1.43	1	0	1.47
2	0	1.43	2	0	1.33
3	0	1.25	3	0	1.21
4	0	1.43	4	0	1.30
5	0	1.43	5	0	1.43
6	0	1.43	6	0	1.12
7	0	1.37	7	0	1.08
8	0	1.13	8	0	1.27
9	0	1.31	9	0	1.37
10	0	1.37	10	0	1.30
11	0	1.43	11	0	1.28
12	0	1.47	12	0	1.12
Average	0	1.37	Average	0	1.27

15 Days			20 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants	Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	0.5	1.12	1	1.6	1.43
2	0.3	1.12	2	1.7	1.50
3	1.4	1.25	3	1.5	1.45
4	0.2	1.25	4	2.9	1.50
5	0.8	1.43	5	2.4	1.37
6	0.3	1.43	6	2.8	1.47
7	0.3	1.25	7	2.4	1.53
8	0.4	1.30	8	2.8	1.44
9	0.6	1.25	9	3.1	1.41
10	0.2	1.12	10	2.4	1.37
11	0.4	1.20	11	2.7	1.45
12	0.3	1.25	12	2.9	1.50
Average	0.4	1.24	Average	2.4	1.43

TABLE 5 (Cont.)

Nodulation and Nitrogen Determinations on Inoculated Alfalfa Plants at the End of Different Growth Periods.

25 Days			30 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants	Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	4.2	2.07	1	6.2	2.12
2	4.2	1.77	2	4.8	1.62
3	4.5	2.03	3	5.2	2.30
4	1.9	1.60	4	3.6	2.22
5	4.0	1.82	5	9.5	2.43
6	2.7	1.67	6	3.3	1.76
7	2.6	2.05	7	6.2	2.37
8	4.1	1.87	8	6.7	2.31
9	3.1	2.12	9	6.1	2.20
10	3.6	2.11	10	6.9	2.11
11	2.5	1.77	11	5.7	2.00
12	2.9	1.87	12	5.9	2.07
Average	3.3	1.89	Average	5.8	2.12

35 Days			40 Days		
Test No.	Average No. Nodules	Mgms. Nitrogen in Plants	Test No.	Average No. Nodules	Mgms. Nitrogen in Plants
1	7.1	2.18	1	5.3	2.62
2	8.5	2.43	2	5.7	2.50
3	4.6	2.40	3	7.5	2.75
4	5.4	2.51	4	9.9	2.90
5	3.1	2.06	5	8.3	2.80
6	4.9	2.10	6	7.4	2.77
7	7.4	2.37	7	5.5	2.69
8	8.2	2.46	8	5.9	2.72
9	7.9	2.38	9	6.2	2.60
10	6.9	2.33	10	5.4	2.65
11	5.7	2.20	11	5.9	2.77
12	8.0	2.44	12	8.6	2.90
Average	6.4	2.32	Average	6.8	2.72

TABLE 5a

Nitrogen Determinations on Uninoculated Alfalfa Plants at the End of Different Growth Periods

7 Days		10 Days		15 Days	
Test No.	Mgm. Nitrogen in Plants	Test No.	Mgm. Nitrogen in Plants	Test No.	Mgm. Nitrogen in Plants
1	1.54	1	1.12	1	1.25
2	1.43	2	1.37	2	1.50
3	1.37	3	1.37	3	1.25
4	1.23	4	1.41	4	1.38
5	1.37	5	1.25	5	1.12
6	1.13	6	1.46	6	1.17
7	1.25	7	1.30	7	1.12
Average	1.37	8	1.27	8	1.15
		Average	1.31	Average	1.24

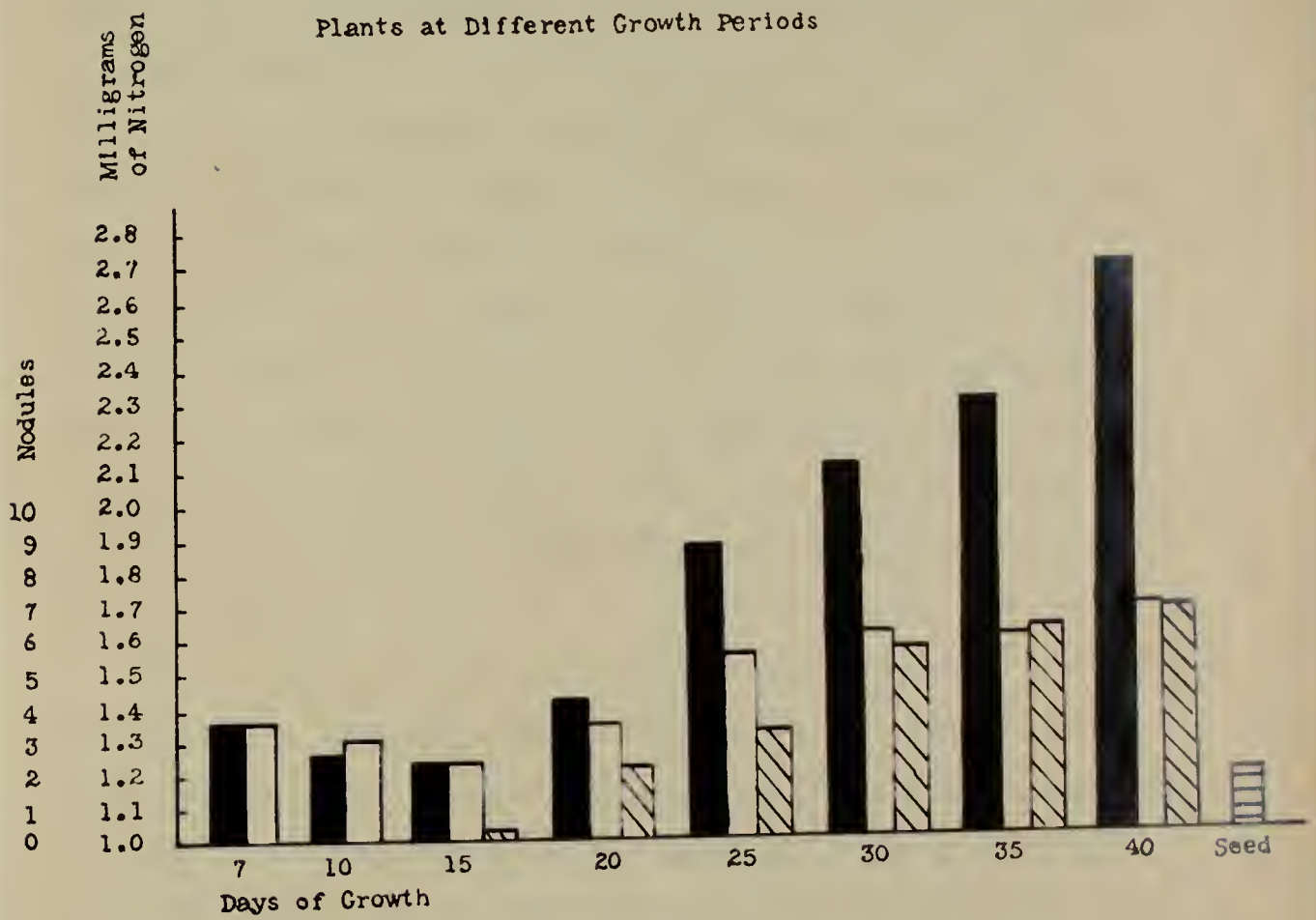
20 Days		25 Days		30 Days	
Test No.	Mgms. Nitrogen in Plants	Test No.	Mgms. Nitrogen in Plants	Test No.	Mgms. Nitrogen in Plants
1	1.50	1	1.58	1	1.61
2	1.62	2	1.52	2	1.59
3	1.62	3	1.68	3	1.74
4	1.25	4	1.43	4	1.67
5	1.37	5	1.65	5	1.64
6	1.25	6	1.56	6	1.54
7	1.55	Average	1.57	Average	1.63
8	1.25				
9	1.10				
10	1.15				
Average	1.36				

35 Days		40 Days	
Test No.	Mgms. Nitrogen in Plants	Test No.	Mgms. Nitrogen in Plants
1	1.54	1	1.70
2	1.70	2	1.66
3	1.65	3	1.69
4	1.59	4	1.75
5	1.61	5	1.71
6	1.59	6	1.70
Average	1.61	Average	1.70

Plate 4 presents a picture of the averaged results of the various tests of each growth period. The solid columns represent the average amount of nitrogen in the inoculated plants. The outlined columns represent the average amount of nitrogen in the uninoculated plants. The cross-hatched columns represent the average number of nodules.

PLATE 4

Average of All Nitrogen Determinations on Alfalfa Plants at Different Growth Periods



The table and graph again indicate that the methods and technique are accurate and efficient for demonstrating minute variations in nitrogen content of the plants.

This plate shows that the first measurable increases in the nitrogen content of inoculated alfalfa plants occur between the 15th and 20th days of growth, which is somewhat earlier than in clover plants. Nodules are visible between the 10th and 15th days, and a short period of time occurs between the appearance of nodules and nitrogen increase in the plant. The increase in nitrogen in alfalfa occurs at about the same rate as in clover plants. This clearly demonstrates that there is little increase in the number of nodules on the plants after 30 days of growth. Referring to Graph 4, we see that there is no appreciable increase in nodulation on clover plants after 40 days of growth. This tends to support the conclusions of Perkins (1925), that there is a period in the growth of legume plants when nodulation reaches a maximum. He places this period at 25-50 days.

From the results of the above experiments it was determined that at least 40 days of plant growth were necessary to demonstrate differences in the nitrogen fixing powers of various strains of Rhizobium leguminosarum.

The results further show that the methods and technique are accurate and efficient for measuring minute increases of plant nitrogen. The perfected technique is to be applied to a study of the nitrogen fixing abilities of several commercial strains of Rhizobium leguminosarum in the part of the project to follow.

The Application of the Technique Already Formulated for Testing the Nitrogen Fixation Powers of Commercial Cultures of Rhizobium Leguminosarum.

The time available for experimental work obviously necessitates limiting the number of cultures to be examined for nitrogen-fixation activity. The following cultures were selected as representative samples of commercial cultures sold in Massachusetts.

Clover	Alfalfa
#63	#61
#64	#62
#75	#89
#77	#74
#69	#68
#70	#101
#80	
#100	

Cultures 100 and 101 are State Experiment Station products, the others are commercially prepared materials. Two experiments were conducted with these cultures.

Experiment #1

The plants were placed on the top floor of the Bacteriology building during their growth period. This experiment was conducted during the Summer and early Fall seasons. The room was so lighted and ventilated that outdoor conditions were closely approximated.

In table 6 is tabulated the average number of nodules per plant and the net increase in nitrogen due to bacterial activity.

TABLE 6

Experiment 1

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of *Rhizobium Leguminosarum* for Red Clover.

No. 63			No. 64		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	1.9	0.37	1	0.2	0.12
2	0.1	0.24	2	0.1	0.49
3	2.1	0.25	3	0.0	0.12
4	2.2	0.25	4	0.0	0.74
5	2.1	0.45	5	0.1	0.12
6	2.0	0.25	6	0.0	0.27
Average	1.7	0.30	Average	0.06	0.31

No. 77			No. 75		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	10.3	1.12	1	7.1	0.99
2	8.8	1.09	2	3.7	0.74
3	9.8	1.24	3	3.9	0.74
4	8.8	1.29	4	4.1	0.79
5	6.3	1.82	5	7.0	1.07
6	7.2	1.37	6	6.0	1.03
Average	8.5	1.32	Average	5.3	0.89

No. 69			No. 70		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	0.3	0.12	1	5.0	1.09
2	0.3	0.00	2	5.1	1.37
3	3.3	0.14	3	4.1	0.97
4	1.9	0.49	4	3.9	1.12
5	1.0	0.37	5	4.6	1.37
6	2.5	0.50	6	4.3	1.27
Average	1.5	0.27	Average	4.5	1.19

No. 80			No. 100		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	3.9	0.93	1	9.1	1.49
2	4.7	0.84	2	6.3	0.97
3	2.4	0.49	3	4.1	1.37
4	1.1	0.37	4	8.0	1.48
5	5.4	0.47	5	7.0	1.37
6	4.0	0.53	Average	6.9	1.33
Average	3.5	0.60			

Controls	
Test No.	Total N.
1	1.07
2	1.32
3	0.86
4	1.07
5	0.95
6	1.16
7	1.49
Average	1.13

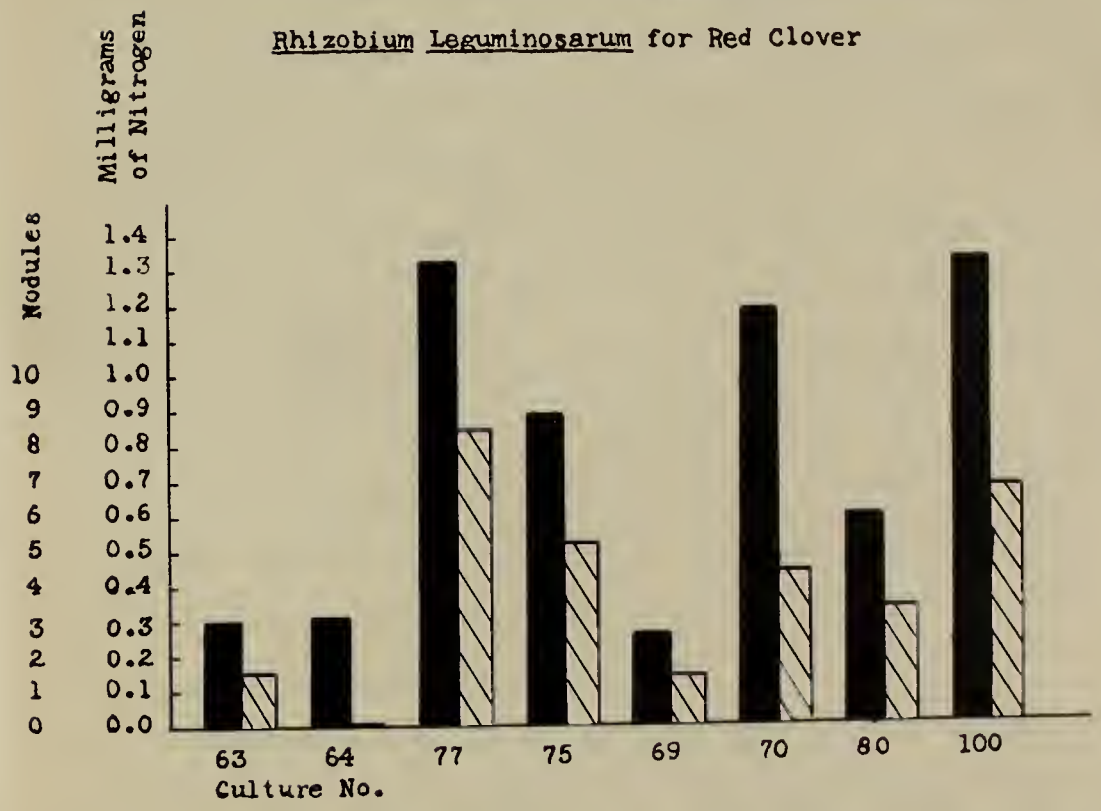
The last chart in Table 6 presents the results of the determinations upon the control (uninoculated) plants. The average nitrogen content of 10 plants is 1.13 mgms., an increase of 0.23 mgm. above the average content of 10 seeds.

Plate 5 presents the averaged results of the individual determinations. It is evident that there is considerable variation in the amounts of nitrogen fixed by the different cultures. The solid columns represent the average amount of nitrogen in the inoculated plants and the cross-hatched columns represent the average number of nodules.

PLATE 5

Experiment 1

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of *Rhizobium Leguminosarum* for Red Clover



If a comparison of culture quality is made by the existing methods. No. 77 proves to be most efficient for increasing the nitrogen content of the plant. This culture produces a higher average amount of nodulation than any of the other cultures. Culture No. 100 would be placed below No. 77 in this quality. By the writer's methods, which makes a quantitative measurement of the amount of nitrogen actually fixed, culture No. 100 is as active, if not slightly more so, than No. 77.

Existing methods cannot accurately differentiate between the nitrogen fixing abilities of cultures Nos. 75, 70 and 80. All produce approximately the same amount of nodulation; but an actual measurement of fixed nitrogen shows a distinct difference in the efficiency of the cultures. Although culture No. 70 produces a slightly less amount of nodulation than No. 75, it is much more active in the fixation of nitrogen. Cultures Nos. 75 and 80 possess an almost equal amount of infectibility, but the latter's fixing ability is much less powerful.

Methods now in vogue for testing cultures would classify Nos. 69 and 63 as "poor" and 64 "worthless". The method of testing proposed by the writer shows that No. 64 is as active in fixing nitrogen as either culture No. 63 or No. 69.

Although the results of this experiment show that there is a relationship between the number of nodules and the nitrogen fixing power of a culture, the relationship is more indirect than direct. Nodulation cannot be accurately used as an index of the relative nitrogen fixing properties of several cultures. In other words, existing methods do

not accurately determine the true quality of commercial legume cultures.

Table 7 presents the results of the initial experiment with alfalfa cultures.

TABLE 7

Experiment 1

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of *Rhizobium Leguminosarum* for Alfalfa.

No. 61			No. 62		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	0.5	0.01	1	0.0	0.01
2	0.2	0.00	2	0.0	0.22
3	0.0	0.43	3	0.3	0.14
4	0.0	0.18	4	0.3	0.22
5	0.0	0.10	5	0.0	0.26
6	0.1	0.26	6	0.2	0.10
Average	0.1	0.16	7	0.1	0.10
			Average	0.1	0.15

No. 89			No. 74		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	2.3	0.93	1	0.6	0.30
2	3.2	0.68	2	0.9	0.01
3	3.8	0.84	3	2.2	0.43
4	4.0	0.68	4	3.3	0.93
5	4.2	0.68	5	3.5	0.43
6	4.1	0.86	6	4.9	0.55
Average	3.6	0.77	7	3.6	0.99
			Average	2.7	0.52

No. 68			No. 101		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	3.9	0.84	1	5.3	1.06
2	3.7	0.51	2	5.7	0.94
3	5.0	0.76	3	7.5	1.19
4	4.0	0.59	4	9.1	1.34
5	3.8	1.01	5	7.3	1.05
6	4.0	0.89	Average	6.9	1.11
Average	4.0	0.76			

Controls

Test No.	Total N. (Mgms.)
1	1.77
2	1.82
3	1.75
4	1.37
5	1.25
6	1.50
Average	1.57

The last chart in the table shows the nitrogen content of the 10 control plants. After 40 days of growth the average nitrogen content of 10 uninoculated plants was 1.57 mgms. Although this figure is higher than that for 10 clover plants, the average nitrogen content of 10 alfalfa seeds is 0.30 mgm. more than that of 10 clover seeds. An average of 1.57 mgm. of nitrogen in the plants is an increase of 0.37 mgm. over the seed. The corresponding increase in the clover plants was 0.29 mgm. The difference between the results is only 0.08 mgm. This indicates that the source of the increase is probably the same in both cases.

Plate 6 shows the averaged results of this experiment. The solid columns represent the average amount of nitrogen in the inoculated plants and the cross-hatched columns represent the average number of nodules.

PLATE 6

Experiment 1

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of Rhizobium Leguminosarum for Alfalfa

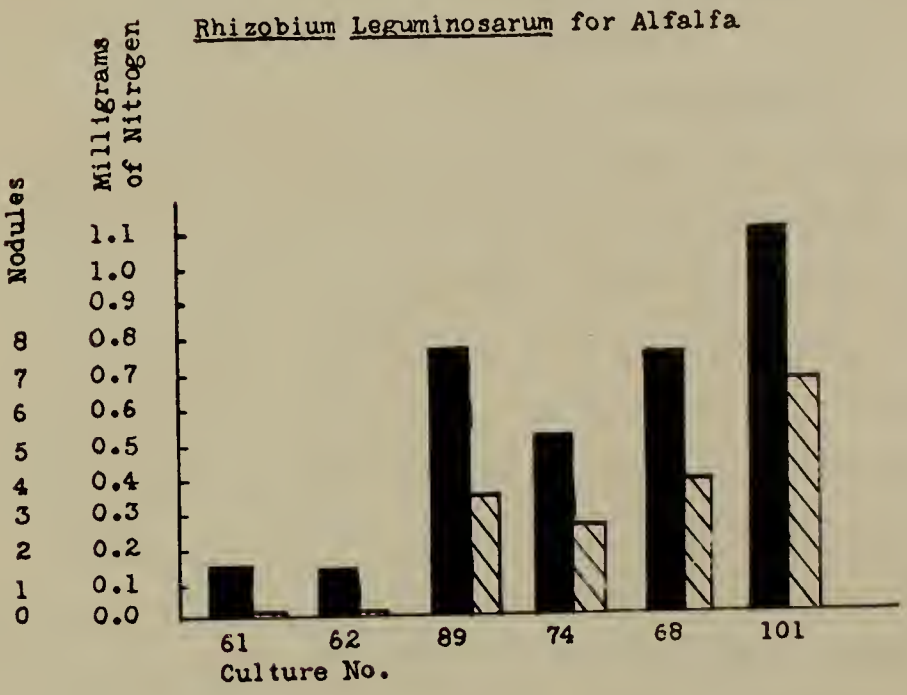


Plate 6 indicates that there is also a variation in the nitrogen-fixing powers and the infectiveness of the alfalfa cultures. The results show that culture No. 101 is the most active in nitrogen-fixation and nodulative power. Existing methods would not be in error in this case. Cultures Nos. 89 and 68 produce almost equal amounts of nodulation and fix practically the same amounts of nitrogen. Although culture No. 74 produces only slightly less nodulation than the above two cultures, it is much weaker in nitrogen-fixing ability. Cultures Nos. 61 and 62 can by all testing methods be classed as "very poor".

It is interesting to note that clover cultures Nos. 63 and 64 and alfalfa cultures Nos. 61 and 62 were all prepared by the same commercial laboratory. It is apparent that this laboratory is selling a strain of symbiotic nitrogen-fixing bacteria of very poor quality. In the quantitative plating experiments these same cultures were also of poor quality in respect to total numbers of viable bacteria. Regulatory measures should be adopted to eliminate such cultures from the market.

Cultures Nos. 100 and 101, which show the most active nitrogen-fixation abilities in this experiment, were the products of the Wisconsin Experiment Station. This Station has for several years been engaged in studies on the strain characteristics of legume bacteria. Probably these cultures are transplants of physiologically active strains.

Experiment #2

The second experiment with clover and alfalfa cultures was almost

a duplication of the first. The plants were placed in the greenhouse during their growth period and the experiment was conducted during the late Fall and early Winter seasons. The object of this duplication was to determine the consistancy of the nitrogen-fixing powers of the various cultures under different growth conditions, and to determine the efficiency and accuracy of the technique. Atmospheric conditions in the greenhouse were necessarily artificial; the temperature was kept at approximately 25°C.

Table 8 presents the results of the duplicate experiment upon the clover cultures.

TABLE 8

Experiment 2

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of *Rhizobium Leguminosarum* for Red Clover

No. 63			No. 64		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	0.1	0.33	1	0.1	0.29
2	0.0	0.27	2	0.6	0.21
3	0.0	0.28	3	0.4	0.27
4	0.0	0.40	4	0.3	0.15
5	0.2	0.37	5	0.2	0.00
6	0.2	0.25	6	0.0	0.22
Average	0.09	0.31	Average	0.2	0.19

No. 77			No. 75		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	8.2	1.24	1	6.3	0.86
2	9.0	1.01	2	3.0	0.61
3	8.6	1.41	3	4.0	0.91
4	10.0	1.21	4	4.4	0.86
5	9.0	1.28	5	4.0	0.87
6	9.1	1.15	6	2.9	0.71
Average	8.9	1.21	7	4.0	0.91
			Average	4.4	0.81

No. 69			No. 70		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	2.0	0.26	1	4.6	0.95
2	1.5	0.33	2	3.6	1.06
3	1.9	0.16	3	4.0	0.81
4	1.0	0.21	4	5.0	1.13
5	3.0	0.55	5	4.0	0.97
6	2.8	0.35	6	4.3	1.21
Average	2.0	0.31	Average	4.3	1.02

No. 80			No. 100		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	5.0	0.71	1	7.5	1.61
2	4.4	0.67	2	8.0	1.55
3	5.6	0.81	3	5.0	1.41
4	2.0	0.51	4	5.3	1.49
5	2.3	0.65	Average	6.4	1.51
Average	3.7	0.67			

Controls	
Test No.	Total N. (Mgms.)
1	1.41
2	1.16
3	1.41
4	1.25
5	1.50
6	1.14
7	0.89
8	1.20
9	1.00
10	0.96
Average	1.19

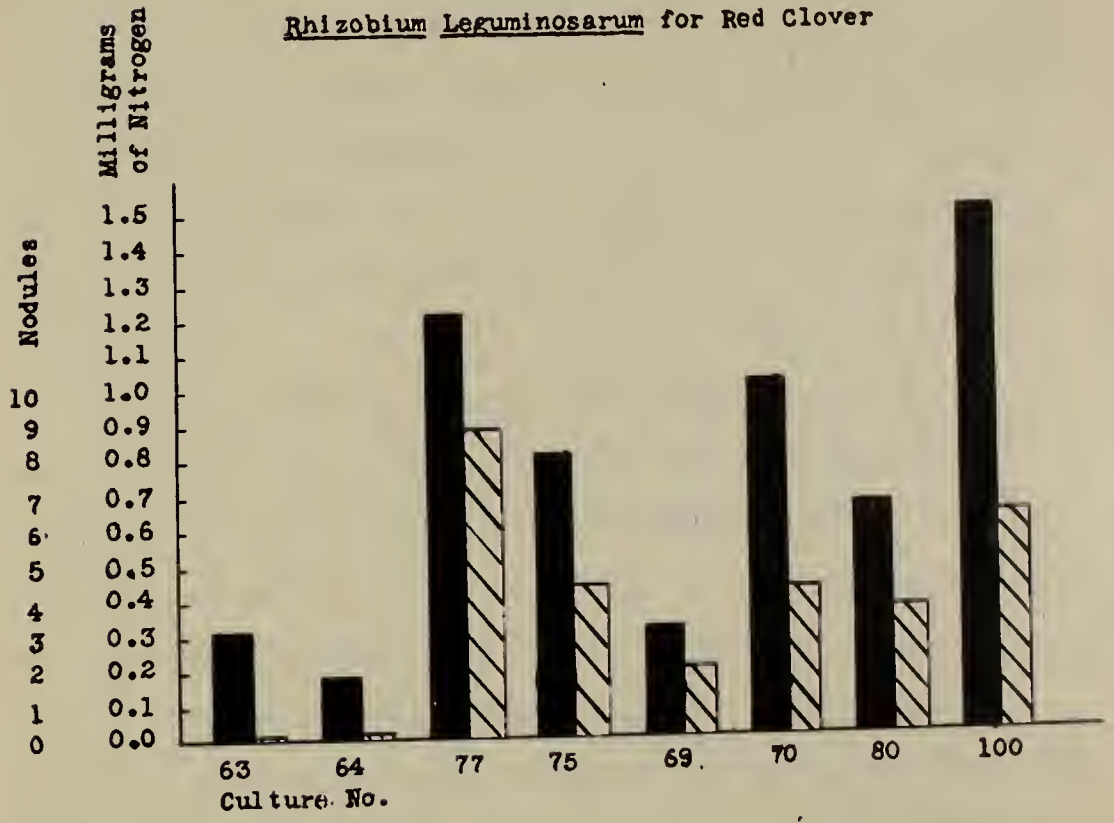
The last chart of this Table gives the results of the nitrogen determinations upon the uninoculated plants. In this experiment the average nitrogen content was 1.19 mgms., an increase of 0.06 milligram over the corresponding results in the first experiment. With the results so closely related, the fact is emphasized that the source of this increase is the same in all cases.

Plate 7 presents a picture of the averaged results. The solid columns represent the average amount of nitrogen in the inoculated plants and the cross-hatched columns represent the average number of nodules.

PLATE 7

Experiment 2

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of *Rhizobium Leguminosarum* for Red Clover

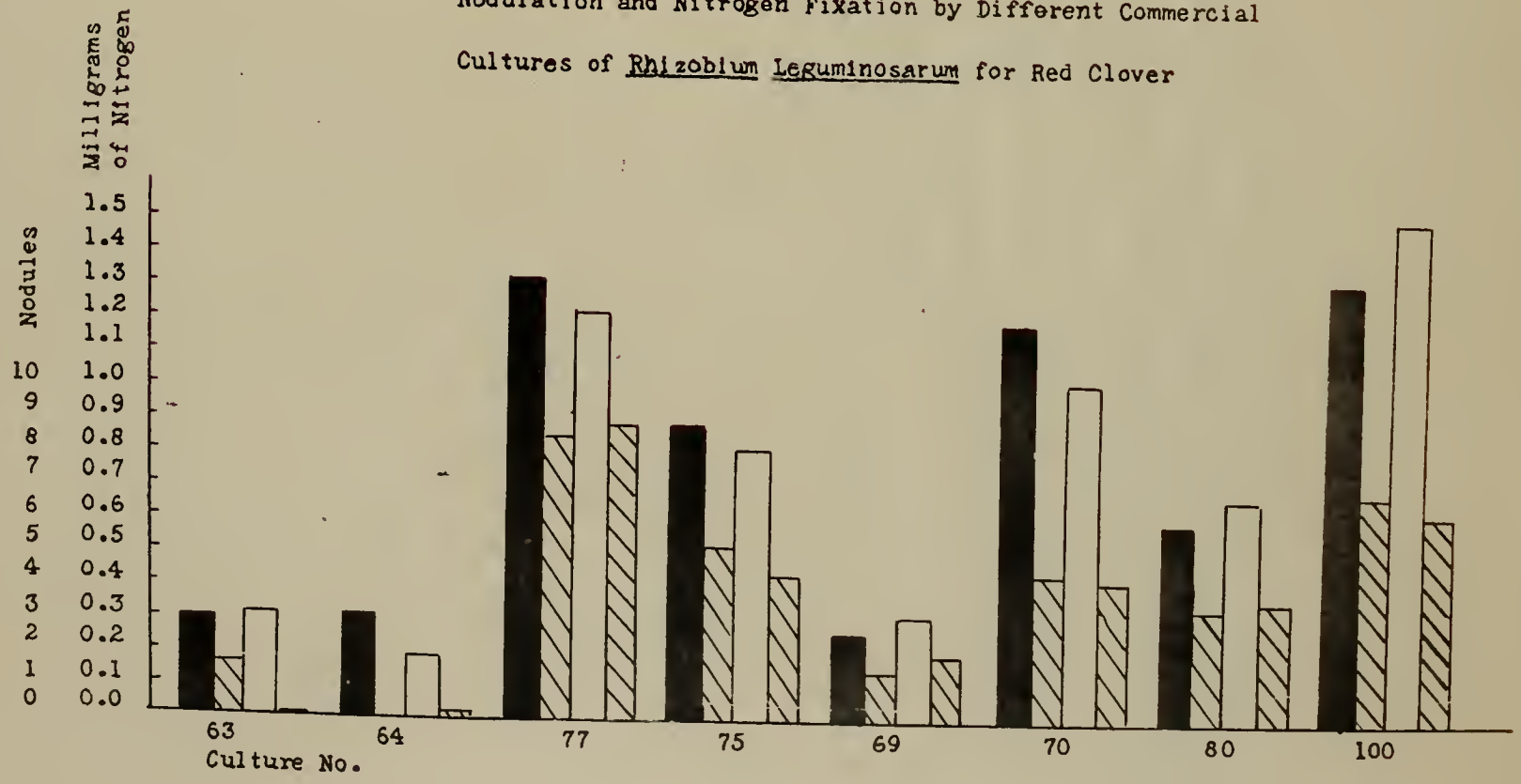


Culture No. 77 again produces the greatest amount of nodulation, with culture No. 100 in the same relative position, as in the former experiment with these cultures. The writer's method reveals that the latter culture is more active in fixing nitrogen than the former. Cultures Nos. 75, 77, and 80 again produce almost equal amounts of nodulation and existing methods could not accurately classify them in the order of their nitrogen fixing powers. The writer's method does this very easily. Culture No. 70 is the most active of the three, No. 75 next and culture No. 80 the weakest. The relationship between cultures Nos. 69 and 63 remains the same in this experiment as existed in the first.

Plate 8 demonstrates a comparative picture of the results of the two experiments with the clover cultures. The solid columns represent the average amount of nitrogen in the inoculated plants in the first experiment. The outlined columns represent the average amount of nitrogen in the inoculated plants in the second experiment. The cross-hatched columns represent the average number of nodules on the plants - the results of the first experiment immediately to the right of the solid columns, and the results of the second experiment immediately to the right of the outlined columns.

PLATE 8

Comparison of Results of Experiments 1 and 2 Concerning Nodulation and Nitrogen Fixation by Different Commercial Cultures of Rhizobium Leguminosarum for Red Clover



It can be seen that there is only a slight difference in the amounts of nitrogen fixed by the different cultures in the two experiments. From the data already at hand the results of both experiments with red clover indicate that there is a very close agreement in results although conducted under different conditions of plant growth. The data thus far presented in this project sufficiently indicates that the procedure and technique are efficient and accurate.

Table 9 gives the results of the duplicate experiment with the alfalfa cultures.

TABLE 9

Experiment 2

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of *Rhizobium Leguminosarum* for Alfalfa.

No. 60			No. 61		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	0.1	0.00	1	0.1	0.03
2	0.2	0.00	2	0.1	0.00
3	0.3	0.00	3	0.1	0.00
4	0.2	0.00	4	0.4	0.00
5	0.0	0.00	5	0.0	0.00
6	0.1	0.00	6	0.0	0.00
Average	0.1	0.00	7	0.3	0.00
			8	0.1	0.24
			Average	0.1	0.00

No. 89			No. 68		
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Average No. Nodules	Net Gain (Mgms.)
1	2.5	0.58	1	3.5	0.68
2	6.5	0.49	2	3.7	0.74
3	3.8	0.49	3	3.0	0.60
4	2.7	0.58	4	2.9	0.64
5	2.0	0.41	5	3.2	0.57
6	3.0	0.00	6	4.1	0.80
Average	3.2	0.42	Average	3.4	0.67

No. 101			Controls	
Test No.	Average No. Nodules	Net Gain (Mgms.)	Test No.	Total N. (Mgms.)
1	3.1	0.55	1	1.87
2	3.0	0.34	2	1.57
3	5.0	0.76	3	1.63
4	5.0	0.85	4	1.69
5	5.9	1.08	5	1.70
Average	4.4	0.71	6	1.53
			Average	1.66

Considerable difficulty was experienced in obtaining optimum plant growth under greenhouse conditions. Future work with cultures specific for alfalfa plants should be conducted at a time of year when approximately natural conditions can be obtained.

Plate 9 presents a picture of the averaged results of this experiment. The solid columns represent the average amount of nitrogen in the inoculated plants and the cross-hatched columns represent the average number of nodules.

PLATE 9

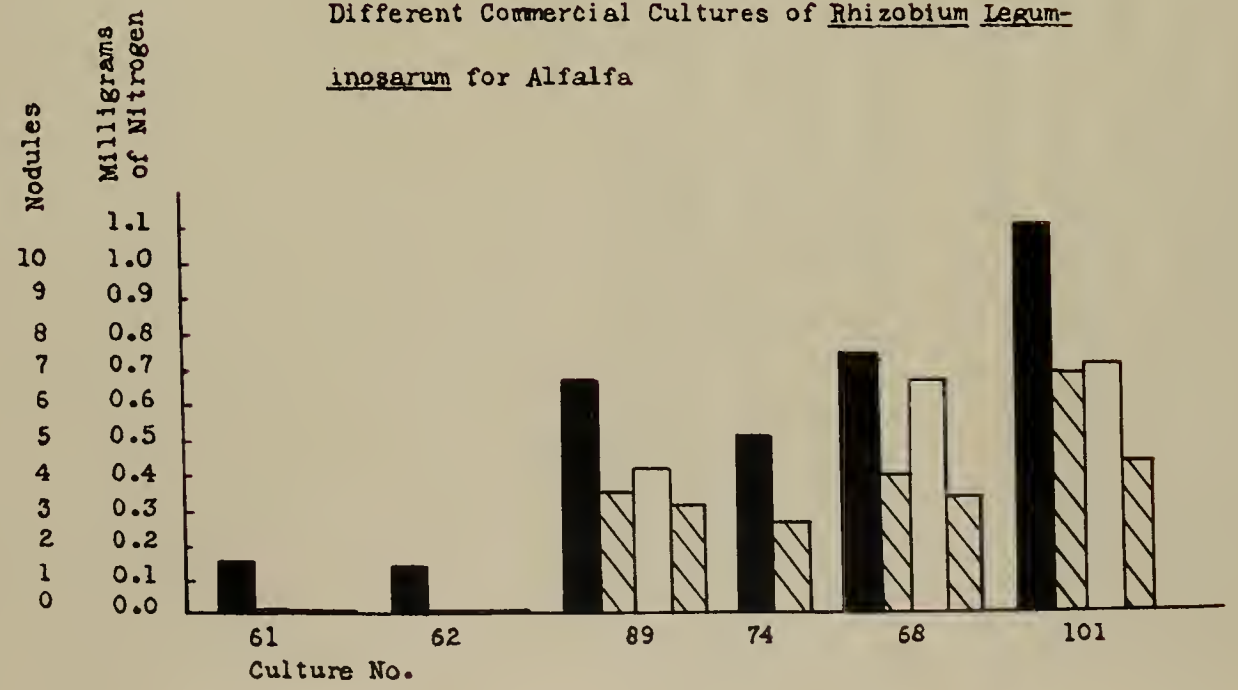
Experiment 2

Correlation Between Nodulation and Net Nitrogen Gain by Different Commercial Cultures of Rhizobium Leguminosarum for Alfalfa

Compared with the first experiment with alfalfa plants it will be seen that there is a considerable decrease in the amounts of nitrogen fixed by the cultures (Plate 10) although the relative nitrogen-fixing powers of the cultures are the same in both experiments.

PLATE 10

Comparison of Results of Experiments 1 and 2 Concerning Nodulation and Nitrogen Fixation by Different Commercial Cultures of Rhizobium Leguminosarum for Alfalfa



Culture No. 101 is the most active both in infectibility and nitrogen fixation. Cultures Nos. 61 and 62 show no nitrogen accumulation in this experiment. There is very little difference in the extent of infectibility between cultures 68 and 79, but the former is the more active in fixing nitrogen. The results of the two experiments are comparable.

In both clover and alfalfa cultures there is a wide variation in the nitrogen-fixing and nodulative powers of the various strains tested. Existing method should not be used to estimate the value of commercial cultures in forms of nitrogen fixing ability, but the methods suggested in this paper may be employed to accurately classify cultures in terms of nitrogen fixing powers.

SUMMARY

1. The commercial legume cultures examined in this study were, with few exceptions, pure cultures of Rhizobium leguminosarum. In the contaminated cultures, extraneous forms were present in relatively small numbers.

2. In the majority of cases the commercial cultures contained a sufficient number of nitrogen-fixing bacteria to insure adequate seed inoculation.

3. The questionable quality of one group of commercial cultures emphasizes a need for laboratory control of production and distribution.

4. Nodulation and nitrogen-fixation in the leguminous plants do not appear simultaneously. There is an interval of about 5 days between the appearance of nodules and the first indication of nitrogen increase in the plant tissue.

5. Existing methods for testing commercial legume cultures are not sufficiently delicate to classify accurately the nitrogen-fixing power of various strains of Rhizobium leguminosarum.

6. A method is suggested for the accurate determination of the nitrogen-fixing power of various strains of Rhizobium leguminosarum. Quantitative measurement of nitrogen increase in the tissue of inoculated plants is used as an index of culture efficiency.

7. The new method is applicable to laboratory testing of commercial legume cultures.

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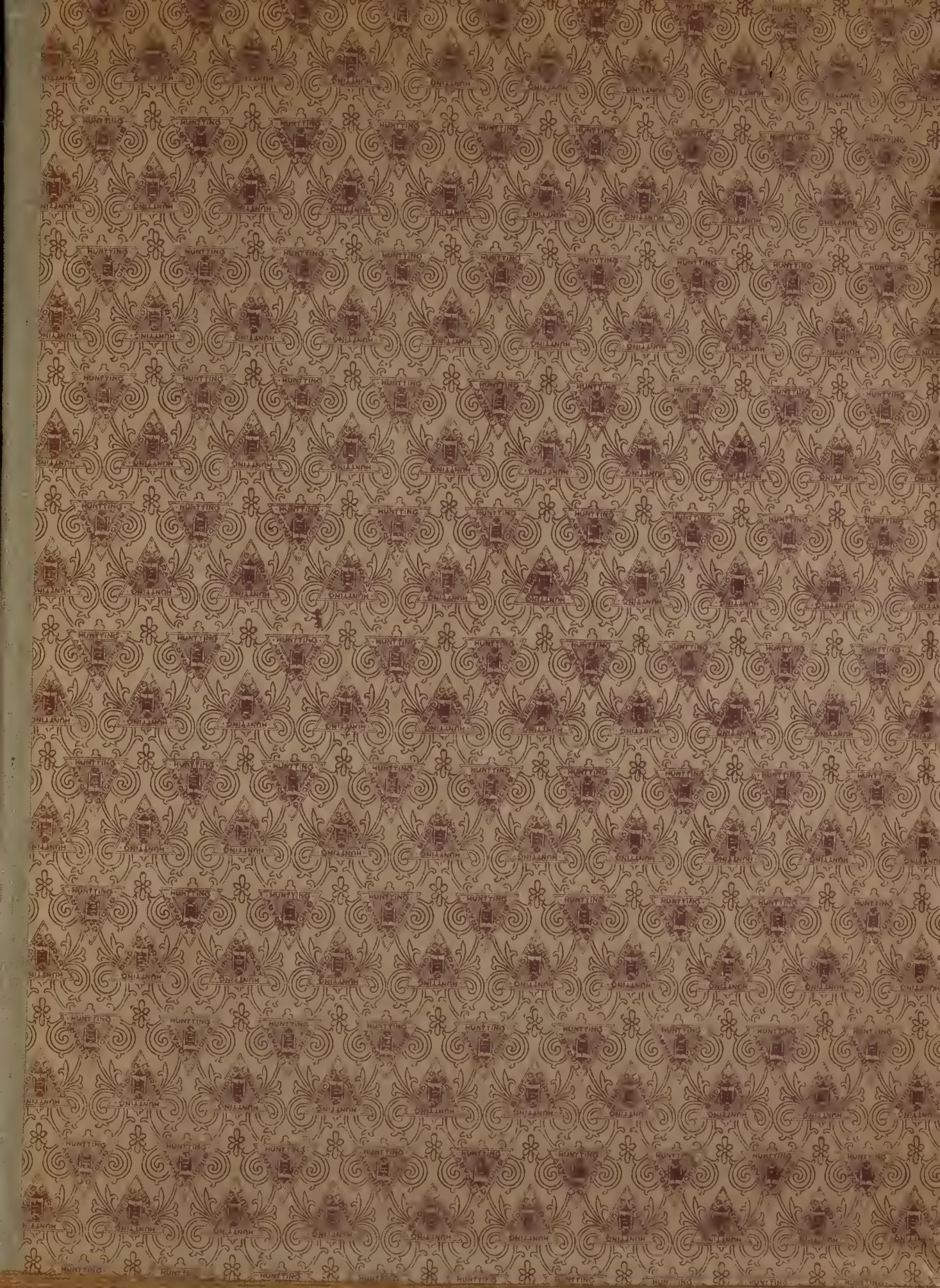
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